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Close Association of Verotoxin (Shiga-Like Toxin) Production with Enterohemolysin Production in Strains of *Escherichia coli*

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Sixty-four verotoxin-producing (VT^+) *Escherichia coli* strains were analyzed for VT1- and VT2-specific DNA sequences and for production of hemolysin. Strains of human origin were of the following serotypes: O157:H7 or H−, O111:H8 or H−, O26:H11, O114:H4, and rough:H7. Strains of serotypes O157:H7, O113:H21, O116:H21, and rough:H− were from cattle, while those of serotype O139:K12:H1 were from pigs. All 64 isolates carried either VT1 or VT2 or both genes. Sixty of the strains (93.8%) were hemolytic (Hly^+). The three O139:K12:H1 strains examined produced α-hemolysin, as shown by their reaction with the α-hemolysin-specific monoclonal antibody h2A and by DNA hybridization with an α-*hly* gene probe. The remaining 57 Hly^+ strains (95%) produced a different type of hemolysin (enterohemolysin), which is genetically and serologically unrelated to α-hemolysin. The two types of hemolysin are further distinguished by the appearance of the lysis zone on blood agar and by the time interval for the detection of hemolysis. In contrast to α-hemolysin, enterohemolysin can be detected only on blood plates containing washed erythrocytes. The frequent association of enterohemolysin with verotoxin production (89%) makes it useful as an epidemiological marker for rapid and simple detection of potential VT^+ *E. coli*.

Vero(Shiga-like)toxin-producing *Escherichia coli* (VTEC) strains constitute a new group of pathogens causing hemorrhagic colitis and hemolytic uremic syndrome in humans (9, 17, 28, 29). Verotoxin-producing (VT^+) strains have also been isolated from healthy pigs and cows as well as pigs and cows with diarrhea (9, 21, 22, 27, 35) and from contaminated foodstuffs of animal origin (9, 29). Although *E. coli* of serotype O157:H7 has frequently been found to be associated with hemorrhagic colitis and hemolytic uremic syndrome, the VTEC group comprises *E. coli* strains of great serological diversity (6, 9, 12, 21, 30, 35). Serotyping of O and H antigens, biotyping, and the development of selective media containing sorbitol have been useful in routine screening for O157:H7 and H− strains but cannot be used for the detection of VT^+ strains with other fermentative properties (6, 14, 18, 20, 30). Other screening systems employ the direct detection of verotoxins (Shiga-like toxins) by using antitoxin monoclonal antibodies (16, 36) or DNA probes which are specific for the genetic sequences encoding verotoxins (VT phenotype) or Shiga-like toxins (SLT phenotype) (5, 24, 31, 33, 35). These methods are very specific and reliable for the detection of all potential VT^+ or Shiga-like toxin-producing (SLT^+) strains, but they are presently not commonly used routinely in clinical laboratories.

Recently, we have described a new type of *E. coli* hemolysin, called enterohemolysin, in some strains belonging to the classical enteropathogenic *E. coli* (EPEC) O groups 26 and 111 (4). Since strains belonging to these groups are also described as potential VTEC strains (35, 36), we studied hemolysin production in a large number of serologically diverse VT^+ *E. coli* strains and found an association between enterohemolysin and verotoxin production in 89% of *E. coli* strains belonging to nine different serotypes. Production of α-hemolysin was detected only in porcine VT^+ strains of serotype O139:K12:H1. We conclude that enterohemolysin may be useful as an epidemiological marker for rapid selection of potential VT^+ strains of *E. coli*.

MATERIALS AND METHODS

Bacteria. Sixty-six *E. coli* strains of different sero-biotypes were investigated for production of verotoxin and hemolysin (Hly phenotype). The strains were isolated from humans and animals and came from laboratories in the United States, Canada, New Zealand, France, Denmark, the United Kingdom, and the Federal Republic of Germany. A complete strain list with further references is presented in Table 1. The VT^+ O26 and O111 strains listed in Table 1 were selected from a greater number of these serotypes which were not VT^+. Strains U4-41 (O4:K3:H5) and C4170 (O78:H−) served as control strains for high and low α-hemolysin production, respectively (also listed in Table 1). The study furthermore included 267 fecal *E. coli* strains from healthy infants which were previously examined for hemolysin production (4) and 45 enterotoxigenic *E. coli* (ETEC) strains which were isolated in India, Peru, and the Federal Republic of Germany (2; this work). DNA-DNA hybridization experiments used the *E. coli* K-12 strains WAF100, carrying the *hly* recombinant plasmid pSF4000 (38) (donated by D. A. Low, Stanford University, Stanford, Calif.), and 6OR746 and 6OR363, carrying recombinant plasmids encoding either VT1 (NTP705)- or VT2 (NTP707)-specific sequences (39, 40) (donated by H. Smith, Central Public Health Laboratory, London, United Kingdom).

Media. Media for routine cultivation of bacteria were described previously (3, 4). For detection of hemolysis, bacteria were grown on tryptose blood agar base (Difco Laboratories, Detroit, Mich.) supplemented with 10 mM $CaCl_2$ and 5% defibrinated sheep blood (Oxoid, Wesel, Federal Republic of Germany) washed three times in phosphate-buffered saline, pH 7.2. In Copenhagen, washed blood

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TABLE 1. Bacterial strains and properties

Strain	Serotype	Origin[a] and yr isolated	Source[b] or reference	Biotype[c]	Hemolysin type[d]	Immunoblot with h2A	DNA hybridization with gene probes specific for:			Vero cell test
							α-*hly*	VT1	VT2	
C1403-83	O157:H7	(h)	UK	574	EntHly	−	−	+	+	+
E32511	O157:H−	(b) Great Britain	32	576	EntHly	−	−	−	+	+
C999-87	O157:H7	(a) Denmark, 1987	HF	574	EntHly	−	−	−	+	+
C1011-87	O157:H7	(a) Denmark, 1987	HF	576	EntHly	−	−	−	+	+
C7-88	O157:H−	(a) Denmark, 1988	HF	574	EntHly	−	−	+	+	+
CL56	O157:H7	(c) Canada	HUS	554	EntHly	−	−	+	+	+
CB429	O157:H7	(d) Berlin, FRG, 1987	Cattle, F	570	EntHly	−	−	−	+	+
CB430	O157:H7	(d) Berlin, FRG, 1987	Cattle, F	570	EntHly	−	−	−	+	+
A9047-CS1	O157:H7	(f) CDC, Atlanta	UK	573	EntHly	−	−	+	+	+
A9167-1	O157:H7	(e) CDC, Atlanta	6	574	EntHly	−	−	+	+	+
A9218-C1	O157:H7	(f) CDC, Atlanta	UK	574	EntHly	−	−	+	+	+
EDL933	O157:H7	(f) CDC, Atlanta	UK	574	EntHly	−	ND[e]	+	+	+
CL40	O157:H7	(c) CDC, Atlanta	UK	574	EntHly	−	−	+	+	+
A8959-C7	O157:H7	(f) CDC, Atlanta	UK	574	EntHly	−	−	+	+	+
B1189-1	O157:H7	(f) CDC, Atlanta	UK	474	EntHly	−	−	+	+	+
3149-85	O157:H7	(f) CDC, Atlanta	UK	574	EntHly	−	−	+	+	+
B2576	O157:H7	(f) CDC, Atlanta	UK	574	EntHly	−	−	−	+	+
161-84	O157:H7	(e) CDC, Atlanta	6	577	EntHly	−	ND	+	+	+
3124-85	O157:H7	(e) CDC, Atlanta	6	577	EntHly	−	ND	+	+	+
3230-85	O157:H7	(e) CDC, Atlanta	6	574	EntHly	−	ND	+	+	+
3174-87	O157:H7	(e) CDC, Atlanta	6	764	EntHly	−	ND	−	+	+
3199-85	O157:H−	(e) CDC, Atlanta	6	576	EntHly	−	ND	−	+	+
3205-85	O157:H−	(e) CDC, Atlanta	6	574	EntHly	−	ND	+	+	+
3344-85	O157:H−	(e) CDC, Atlanta	6	574	EntHly	−	ND	+	+	+
3460-85	O157:H−	(e) CDC, Atlanta	6	574	EntHly	−	ND	+	+	+
EDL932	O157:H−	(e) CDC, Atlanta	6	576	EntHly	−	−	+	+	+
3526-87	O157:H7	(e) CDC, Atlanta	6	575	EntHly	−	ND	+	+	+
1271-84	O157:H−	(e) CDC, Atlanta	6	574	EntHly	−	−	−	+	+
3417-86	O157:H7	(e) CDC, Atlanta	6	574	Hly$^-$	−	ND	−	−	−
CB573	O157:H7	(g) FRG, 1987	HF	777	EntHly	−	ND	−	+	+
CB571	O157:H7	(g) FRG, 1987	HF	574	EntHly	−	ND	+	+	+
CB570	O157:H7	(g) FRG, 1987	HF	776	EntHly	−	ND	+	+	+
CB572	O157:H43	(g) FRG, 1988	HF	671	Hly$^-$	−	ND	−	−	−
CB569	O157:H−	(g) FRG, 1987	HF	766	EntHly	−	ND	−	+	+
H19	O26:H11	(b) Great Britain	32	763	EntHly	−	−	+	−	+
IP29580	O26:H11	(h) Palermo, Italy, 1965	4	762	EntHly	−	−	+	−	+
IP29581	O26:H11	(h) Palermo, Italy, 1965	UK	762	EntHly	−	−	+	−	+
CB282	O26:H11	(i) New Zealand	HF	723	EntHly	−	−	+	−	+
CB283	O26:H11	(i) New Zealand	HF	763	EntHly	−	−	+	−	+
CB284	O26:H11	(i) New Zealand	HF	763	EntHly	−	−	+	−	+
CB288	O26:H11	(i) New Zealand	HF	763	EntHly	−	−	+	−	+
CB285	O26:H11	(i) New Zealand	HF	763	EntHly	−	−	+	−	+
CB290	O26:H11	(i) New Zealand	HF	763	EntHly	−	−	+	−	+
CB291	O26:H11	(i) New Zealand	HF	763	EntHly	−	−	+	−	+
CB292	O26:H11	(i) New Zealand	HF	763	EntHly	−	ND	+	−	+
B1540-C2	O111:H−	(f) CDC, Atlanta	UK	274	EntHly	−	−	+	+	+
C3007-86	O111:H−	(e) CDC, Atlanta	UK	274	EntHly	−	ND	+	+	+
C4169	O111:H−	(j) Stuttgart, FRG, 1985	HF (4)	777	EntHly	−	−	+	−	+
CB24	O111:H−	(j) Saarbrücken, FRG, 1985	HF (4)	274	EntHly	−	−	+	−	+
CB168	O111:H−	(j) Bonn, FRG, 1985	HF (4)	254	EntHly	−	−	+	−	+
CB27	O111:H8	(j) Saarbrücken, FRG, 1985	HF (4)	777	EntHly	−	−	+	−	+
C3075/69	O114:H4	(j) Berlin, FRG, 1969	HF	674	Hly$^-$	−	ND	+	−	+
CL8	Rough:H7	(c)	HUS	676	EntHly	−	−	+	+	+
CB462	Rough:H−	(d) Berlin, FRG, 1987	Cattle, F	610	EntHly	−	−	+	−	+
CB463	Rough:H−	(d) Berlin, FRG, 1987	Cattle, F	610	EntHly	−	−	+	−	+
CB425	O113:H21	(d) Berlin, FRG, 1987	Cattle, F	770	Hly$^-$	−	ND	−	+	+
CB426	O113:H21	(d) Berlin, FRG, 1987	Cattle, F	770	Hly$^-$	−	ND	−	+	+
CB427	O113:H21	(d) Berlin, FRG, 1987	Cattle, F	770	Hly$^-$	−	ND	−	+	+

Continued on following page

from sheep kept at the farm of the Statens Seruminstitut was used. For some experiments, blood agar plates containing unwashed sheep blood were used. For the Vero cell test, bacteria were grown in tryptic soy broth (Difco) as described previously (4).

Biotyping. The following sugars or alcohols were used for biotyping: sorbitol, rhamnose, sucrose, raffinose, salicin, sorbose, and dulcitol (E. Merck AG, Darmstadt, Federal Republic of Germany). The strains were also examined for production of lysine and ornithine decarboxylases. The tests

TABLE 1—*Continued*

Strain	Serotype	Origin[a] and yr isolated	Source[b] or reference	Biotype[c]	Hemolysin type[d]	Immunoblot with h2A	DNA hybridization with gene probes specific for:			Vero cell test
							α-*hly*	VT1	VT2	
CB464	O116:H21	(d) Berlin, FRG, 1987	Cattle, F	774	EntHly	−	−	+	+	+
CB465	O116:H21	(d) Berlin, FRG, 1987	Cattle, F	774	EntHly	−	ND	+	+	+
CB466	O116:H21	(d) Berlin, FRG, 1987	Cattle, F	774	EntHly	−	ND	+	+	+
CB467	O?:H18	(d) Berlin, FRG, 1987	Cattle, F	667	EntHly	−	−	+	+	+
CB468	O?:H18	(d) Berlin, FRG, 1987	Cattle, F	667	EntHly	−	−	+	+	+
CB295	O139:K12:H1	(i) New Zealand	Pig, F	276	α-Hly	+	+	−	+	+
CB301	O139:K12:H1	(i) New Zealand	Pig, F	276	α-Hly	+	+	−	+	+
CB302	O139:K12:H1	(i) New Zealand	Pig, F	276	α-Hly	+	+	−	+	+
U4-41[f]	O4:K3:H5	(a) Copenhagen, 1941	HU (26)	ND	α-Hly	+	+	−	−	−
C4170[f]	O78:H−	(j) Saarbrücken, FRG, 1985	HF (4)	ND	α-Hly	+	+	−	−	−

[a] Strains were obtained from the following sources: (a) I. Ørskov and F. Ørskov, Statens Seruminstitut, International Escherichia and Klebsiella Centre, Copenhagen, Denmark; (b) H. Smith, Division of Enteric Pathogens, Central Public Health Laboratory, London, United Kingdom; (c) M. A. Karmali, Department of Bacteriology and Virology, Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada; (d) M. Bülte, Institut für Lebensmittelhygiene, FU Berlin, Berlin, Federal Republic of Germany (FRG); (e) Centers for Disease Control (CDC), Atlanta, Ga., from N. A. Strockbine, Escherichia Shigella Reference Laboratory, Atlanta, Ga.; (f) CDC, Atlanta, from A. D. O'Brien, Uniformed Services University of the Health Sciences, Bethesda, Md.; (g) S. Aleksic, Hygienisches Institut der Freien Hansestadt Hamburg, Hamburg, FRG; (h) L. Le Minor, Service des Entérobactéries, Institut Pasteur, Paris, France; (i) K. A. Bettelheim, Department of Health, National Health Institute, Wellington, New Zealand; (j) strain collection of the Robert Koch-Institute, Berlin, FRG.

[b] HF, Human feces; F, fecal; HUS, hemolytic uremic syndrome; HU, human urine; UK, unknown.

[c] Biotype is expressed by a numerical code as described in Materials and Methods.

[d] EntHly, enterohemolysin; α-Hly, α-hemolysin; Hly⁻, hemolysin negative.

[e] ND, Not done.

[f] Control strain.

were performed as described by Achtman et al. (1). The biotypes were expressed by a numerical code which is a summary of the values given to each positive test in the following way: ornithine decarboxylase, 100; sorbitol, 200; lysine decarboxylase, 400; rhamnose, 10; sucrose, 20; raffinose, 40; salicin, 1; sorbose, 2; and dulcitol, 4. The reactions were read after different periods of incubation by the method of Crichton and Old (8).

Serotyping. Serotyping of *E. coli* strains was performed by standard methods (26).

Immunochemicals and colony immunoblots. For immunoblotting, the monoclonal antibody h2A, which is specific for the *hlyA* gene product (107-M_r [107K] protein) (15), was used (provided by S. Bhakdi, University of Giessen, Giessen, Federal Republic of Germany). An alkaline phosphatase-conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulin G and immunoglobulin M (Jackson Immunoresearch Laboratory, Avondale, Pa.) was used as the second antibody. Naphthol AS-MX phosphate and fast red TR salt (both from Sigma Chemical Co., St. Louis, Mo.) were used for chemical stainings of immunoblots as described before (2). The colony immunoblot method for detection of the *hlyA* gene product was performed as described previously (4).

Other techniques. DNA isolation, agarose gel electrophoresis, preparation of the *hly*-specific gene probe, and Southern blot analysis were performed as described previously (3).

Colony DNA hybridization for detection of VT⁺ strains. Colony blots were prepared on Whatman 541 filters (Whatman International Ltd., Maidstone, United Kingdom) by the method of Maas (19). Gene probes specific for VT1 (750-base-pair [bp] *Hinc*II fragment of plasmid NTP705) (40) and VT2 (850-bp *Ava*I-*Pst*I fragment of plasmid NTP707) (39) were labeled with [^{32}P]dCTP (Amersham) by nick translation. Hybridizations were performed at high stringency as described previously (23) except that the incubation was performed at 42°C.

RESULTS

Detection of VT⁺ strains by the Vero cell test. Of the 66 strains studied (Table 1), 64 produced verotoxins. Only two O157 strains, apart from the two α-hemolysin control strains U4-41 and C4170, were negative for verotoxin in the Vero cell test. The toxigenic potential of VT⁺ strains was generally high. Damage of Vero cells was observed in 10^{-4} dilutions of culture fluid. The porcine VT⁺ isolates were found to be less toxigenic, only causing damage of cells at dilutions of 10^{-3} or less.

Detection of VT1- and VT2-specific sequences by DNA hybridization. All strains, including the 267 fecal *E. coli* isolates from infants, were tested for the presence of VT1- and VT2-specific sequences by colony blot hybridization. All strains which were VT⁺ in the cell assay hybridized with one or both of the two verotoxin gene probes used. The distribution of the VT1 and VT2 gene sequences in the

TABLE 2. Distribution of genes encoding VT1 and VT2 in 64 different VT⁺ strains

Serotype	No. of strains positive		
	VT1	VT2	VT1 + VT2
O26:H11	11	0	0
O111:H8	1	0	0
O114:H4	1	0	0
Rough:H−	2	0	0
O113:H21	0	3	0
O139:K12:H1	0	3	0
O111:H−	3	0	2
O157:H−	0	4	5
O157:H7	0	7	16
O116:H21	0	0	3
O?:H18	0	0	2
Rough:H7	0	0	1

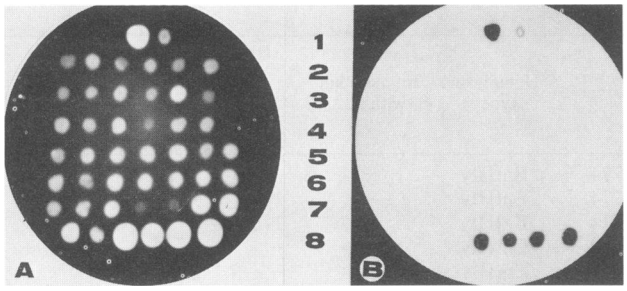

FIG. 1. Colony immunoblots of VT^+ strains with h2A monoclonal antibody. (A) Blood agar plate containing washed sheep blood inoculated with VT^+ *E. coli* strains. The colonies were removed after blotting. (B) Colony immunoblot of plate in panel A. Rows 1 to 8 (from left to right): row 1, U4-41 and C4170 (positive controls for high and low α-hemolysin production, respectively); row 2, C1403-83, E32511, C999-87, C1011-87, 3149-85, and B2576; row 3, EDL933, CL40, A8959-C7, B1189-1, CB468, and A9047-CS1; row 4, A9167-1, A9218-C1, C7-88, CL56, CB429, and CB430; row 5, CL8, B1540-C2, CB27, CB168, CB24, C4169, and IP29580; row 6, IP29581, H19, CB282, CB283, CB284, CB285, and CB288; row 7, CB290, CB291, CB292, CB462, CB463, CB464, and CB465; row 8, CB466, CB467, CB295, U4-41, CB302, and CB301. Strains and properties are listed in Table 1.

strains is shown in Table 2. Of the O157 strains, 65.6% carried both genes, 34.4% were positive for only VT2, and none of the strains was carrying exclusively VT1 sequences. All 11 O26:H11 strains, one O111:H8 strain, three O111:H− strains, and one O114:H4 strain were $VT1^+$ $VT2^-$, while two O111:H− strains were $VT1^+$ $VT2^+$. All combinations were found among the non-O157 bovine VT^+ strains. The three O139 strains from pigs were $VT2^+$ only.

All strains which were VT^- in the cell assay and the 267 fecal *E. coli* strains from infants did not hybridize with either the VT1 or the VT2 probe.

Detection of hemolysis. Sixty of the 64 VT^+ strains produced hemolysin, while the two VT^- strains did not. There were two kinds of lysis zones; one was produced by the two α-hemolysin control strains, the three porcine Hly^+ O139 strains, and 40 α-hemolysin-producing strains from healthy infants. This type of lysis was detectable after 3 h of incubation at 37°C and was also apparent on blood agar plates containing unwashed sheep erythrocytes. The lysis caused by the remaining 57 hemolytic strains was typical for enterohemolysin (in O26 and O111 strains), as described previously (4), except that no diphasic zone was seen; a turbid zone was detected under and around the colonies, but no inner clear zone was seen. Enterohemolysin required overnight incubation of the culture and did not appear when the sheep erythrocytes were not washed; in general, the zones were smaller than those of α-hemolytic *E. coli* strains. When sheep blood from the farm of the Statens Seruminstitut was used, the zones did not look turbid, but the blood also required washing and overnight incubation of the culture for demonstration of hemolysis. Both α-hemolytic and enterohemolytic activities of the strains listed in Table 1 could also be demonstrated when sheep blood was replaced by washed human, ox, guinea pig, or rabbit erythrocytes (4; this work).

Discrimination between α- and enterohemolysin-producing strains by colony immunoblots with h2A. All strains from Table 1 were tested with the monoclonal antibody h2A, which is specific for the *hlyA* gene product, the 107K protein (15). The results are summarized in Table 1 and shown in Fig. 1 for 46 strains. Of the VT^+ strains tested, only the porcine O139:K12:H1 isolates reacted with h2A; all other VT^+ strains were negative. The 40 α-hemolysin-producing strains from healthy infants were previously shown to react positively with h2A (4), as did the control strains U4-41 and C4170.

TABLE 3. Hemolytic activities of non-VTEC *E. coli* strains[a]

Strains[b]	No. of strains		
	α-Hemolysin positive	Enterohemolysin positive	Hemolysin negative
Fecal isolates	40	0	227
EPEC	35	4	198
ETEC	0	0	45

[a] All strains were tested for verotoxins by the Vero cell assay and/or DNA hybridization.

[b] Fecal isolates were collected from healthy infants (4); data for EPEC (3) and ETEC (2) strains are from the literature and this work.

Southern hybridizations with an α-*hly*-specific gene probe. Forty-four representative α-hemolytic and enterohemolytic strains from Table 1 were tested by Southern hybridization for α-hemolysin-specific sequences. For this, plasmid and chromosomal DNA preparations of the strains were examined for hybridization with the *Ava*I A fragment of the *hly* recombinant plasmid pSF4000 (38). The results are listed in Table 1. Hybridization to this α-*hly*-specific probe was only detected with plasmid DNA of the three porcine O139:K12:H1 strains and with chromosomal DNA of strains U4-41 and C4170. All other strains tested were negative for α-*hly*-specific sequences, which indicates that their hemolysin genes do not share homology with the cloned α-hemolysin determinant on pSF4000.

Biotypes. For biotyping, substrates were used that provide good discrimination between unrelated strains (8). Fermentation of sorbitol was tested because it has been found to be useful for detection of O157:H7 and H− strains (6, 11, 14, 20). The results are summarized in Table 1. Fermentation of sorbitol within 48 h of incubation was detected with only 5 of the 34 O157 isolates, including four sorbitol-positive strains isolated in the Federal Republic of Germany. All VT^+ strains belonging to serogroups other than O157 fermented sorbitol promptly. Of the O157 strains, 94% (32 of 34) were positive for ornithine and lysine decarboxylases and 91% (31 of 34) were positive for rhamnose, sucrose, and raffinose fermentation. Seventeen of the O157 strains were dulcitol positive and sorbose negative (biotype 574 in Table 1). Eight of the 11 O26:H11 strains were biotype 763, and all 11 strains were found to be negative for rhamnose and dulcitol, typical for O26:H11 strains (25). Four of the six O111 strains were negative for both ornithine and lysine decarboxylases; the remaining two strains were positive in all nine tests in the biotyping scheme. The rough:H− and the O113, O116, O139, and O? strains all gave identical biotype patterns exclusive for each serotype.

Hemolysin production among non-VTEC fecal *E. coli* isolates. We had previously reported that no enterohemolysin-positive strain was found among 267 fecal *E. coli* strains which originated from 200 healthy infants; however, 40 of these strains (15%) were positive for α-hemolysin (4). In this work, we extended our studies on hemolysin production to EPEC and ETEC *E. coli* strains (3, 4). The results of all these studies are summarized in Table 3. A total of 549 strains were tested. Enterohemolysin was detected in only four O26 VT^- EPEC strains and not in any other group.

Thirty-five (14.8%) of the EPEC strains were positive for α-hemolysin. No hemolysin-positive strain was found in a group of 45 ETEC strains obtained from humans (42 strains), animals (2 strains), or foodstuffs (1 strain).

DISCUSSION

Sixty of 64 (93.8%) verotoxin-producing strains of *E. coli* from various sources and of several serotypes were found to produce hemolysins. Two different types of hemolysins were detected. α-Hemolysin was only produced by porcine VT^+ strains of serotype O139:K12:H1 and was found to be plasmid encoded. Of the VT^+ strains, 89% exhibited a different type of hemolysin unrelated morphologically, serologically, and genetically to α-hemolysin. This type of hemolysin resembled the enterohemolysin we recently found in some classical EPEC O26 and O111 strains and in single isolates belonging to O groups 25 and 121 (4). The detection of enterohemolytic activity was facilitated by comparing blood agar plates containing washed and unwashed sheep blood (4; this work).

Neither standard protocols (34, 37) nor the manufacturers' handbooks for commercially available media recommend washing blood for the preparation of blood agar plates, which might explain why enterohemolytic activity has not been detected previously. The production of enterohemolysin appears to be restricted to only some serological groups of *E. coli*. Thus, enterohemolysin production was not found in a large number of strains belonging to classical EPEC O groups except in some O26 and O111 strains (3, 4; this work) or in 267 fecal *E. coli* strains from healthy human infants (4). In contrast, 32 of 34 (94%) strains of serogroup O157 were found to be enterohemolytic.

These findings, together with the close association found between verotoxin and enterohemolysin production, make enterohemolysin suitable as an epidemiological marker for rapid detection of potential VT^+ *E. coli* strains.

In contrast to enterohemolysin, production of α-hemolysin is quite frequent among human fecal *E. coli* strains, and it predominates in strains causing extraintestinal infections in humans (4, 7, 10, 13). With the methods described here, both types of hemolysins were easy to detect and well discriminated by simple observation of the lysis zones on blood agar plates containing washed and unwashed sheep blood and by the time required for hemolysis to become apparent on blood agar plates.

Despite the close association between VT1 and VT2 and enterohemolysin activity, both functions appear to be expressed by independent mechanisms. This was shown by our observation that more *E. coli* strains of O group 26 were positive for enterohemolysin than for verotoxin production (4; unpublished data) and by the finding that VT^+ strains of O groups 113 and 114 were hemolysin negative. We also found that spontaneous Hly^- derivatives of enterohemolysin-producing VT^+ O26 and O157 strains which were selected on blood agar were not altered in their verotoxigenic activity (data not shown).

The significance of enterohemolysin as a true virulence factor of *E. coli* is not known. Culture fluid of VT^- enterohemolysin-positive O26 strains or cell lysates of these were not found to cause visible damage to cultured Vero, HEp-2, or HeLa cells (4; unpublished data). The finding that enterohemolysin-producing *E. coli* strains are closely associated with diarrheal disease could indicate that it might function as a virulence factor by a hitherto unknown mechanism.

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